# Molecular Diagnostics in an Insecure World

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*Session Keynote Address—*

# Molecular Diagnostics in an Insecure World

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SUMMARY. As of October 2001, the potential for use of infectious agents, such as anthrax, as weapons has been firmly established. It has been suggested that attacks on a nations' agriculture might be a preferred form of terrorism or economic disruption that would not have the attendant stigma of infecting and causing disease in humans. Highly pathogenic avian influenza virus is on every top ten list available for potential agricultural bioweapon agents, generally following foot and mouth disease virus and Newcastle disease virus at or near the top of the list. Rapid detection techniques for bioweapon agents are a critical need for the first-responder community, on a par with vaccine and antiviral development in preventing spread of disease. There are several current approaches for rapid, early responder detection of biological agents including influenza A viruses. There are also several proposed novel approaches in development. The most promising existing approach is real-time fluorescent PCR analysis in a portable format using exquisitely sensitive and specific primers and probes. The potential for reliable and rapid early-responder detection approaches are described, as well as the most promising platforms for using real-time PCR for avian influenza, as well as other potential bioweapon agents.

RESUMEN. Diagnóstico molecular en un mundo inseguro.

A partir de Octubre del año 2001, el uso potencial de agentes infecciosos como el carbunco (ántrax) como armas biológicas ha sido firmemente establecido. Se ha sugerido que ataques en la agricultura de un país pudieran ser una forma preferida de terrorismo o de quebranto económico que no tendría el estigma de infectar y causar enfermedad en humanos. El virus de influenza aviar de alta patogenicidad se encuentra en la lista de los diez agentes más importantes como armas biológicas en agricultura, generalmente después de la fiebre aftosa y del virus de Newcastle, ubicándose al inicio o cerca del inicio de dicha lista. Las técnicas de detección rápida para armas biológicas son una necesidad crítica para una primera respuesta, a la par con el desarrollo de vacunas y antivirales para prevenir la diseminación de la enfermedad. Existen varias metodologías para obtener una detección rápida y temprana de agentes biológicos incluyendo los virus de influenza aviar A. También están en desarrollo varias metodologías nuevas. El enfoque más promisorio es el análisis de PCR fluorescente en tiempo real en un formato portátil utilizando iniciadores y sondas altamente sensibles y específicas. Se describe el potencial de los métodos para la detección rápida, temprana y segura, así como la plataforma más promisoria para utilizar la técnica de PCR en tiempo real para influenza aviar y para otros agentes que pueden potencialmente constituirse en armas biológicas.

Key words: diagnostics, influenza, PCR

Abbreviations: FMD = foot-and-mouth disease; FRT/PCR = fluorescent real-time/PCR; LEADER = lightweight epidemiology advanced detection and emergency response; MALDI-TOF = matrix assisted laser desorption ionization time-of-flight; MS = mass spectrometry; NDV = Newcastle disease virus; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphisms; RAPID = ruggedized advanced pathogen identification device

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Table 1. Diseases and animal pathogens of concern to the Agricultural Research Service, U.S. Department of Agriculture.

| Disease | Agent |
|---|---|
| Foot and mouth disease | Apthovirus |
| Velogenic viscerotropic Newcastle disease | Paramyxovirus type 1, specific strains |
| Highly pathogenic avian influenza | Orthomyxovirus, type A, some subtypes H5 and H7 |
| Hog cholera | Pestivirus |
| Rinderpest | Morbillivirus |
| Contagious bovine pleuropneumonia | Mycoplasma |
| Lumpy skin disease | Poxvirus |
| Blue tongue virus | Orbivirus |
| African horse sickness | Orbivirus |
| African swine fever | Asfivirus |

## CURRENT STATE OF MOLECULAR DIAGNOSTICS

Homeland defense has become a new item on everyone's budget request list—including the agricultural world. According to congressional testimonies by D. A. Henderson (8) and others, rapid detection of introduced biological agents is a critical component in protecting human lives, along with rapid development of vaccines and antimicrobials. While we are all aware that profit margins in poultry production scarcely allow for the kinds of expensive molecular detection equipment that are affordable in the world of human health, the polymerase chain reaction (PCR) as a diagnostic tool has been well established among poultry health professionals for many years (1). Several producers now have their own diagnostic capabilities that include routine PCR analysis for many poultry pathogens. As the market for sophisticated portable detection devices that employ PCR becomes greater, the price of on-site detection for agricultural pathogens will come down. Consequently, it is reasonable to pursue development of detection reagents for high-profile poultry pathogens, particularly the rapidly spreading respiratory pathogens. During an outbreak of a foreign animal disease such as highly pathogenic avian influenza, time is a critical factor in the extent of containment of the disease and the assessment of contamination of surrounding poultry operations and wild bird populations. Fluorescence-based PCR detection can use a single platform for detection of a host of pathogens. Once the fundamental target genes have been identified and sequenced in the laboratory, the designed primers and probes can be transferred directly to the portable machine format. Many commercially available machines can use the same chemistries, and multiple fluorescent wavelengths should eventually allow multiplex analysis for more than one pathogen per reaction. Also, unlike immunologically based detection methods, fluorescent primers and probes can be altered slightly to accommodate known genetic changes, without having to regenerate and revalidate as with serum-based reagents.

## AGRICULTURAL BIOLOGICAL THREAT AGENTS

Although agriculture does not immediately come to mind when one considers biowarfare or bioterrorism, every nation that has had a biological warfare program has had an antianimal and an anticrop component (13,14). Thus a number of potential threats have been identified for both animals and plants. Expert panels have been convened in recent years to determine attributes most likely to contribute to effectiveness of a bioweapon, and oftentimes the agents have then been ranked. Table 1 is a working top ten list of animal pathogens that have been used by the Agricultural Research Service in the last 2 years as a guide for developing detection reagents. At least four of these listed organisms (foot-and-mouth disease [FMD] virus, Newcastle disease virus [NDV], hog cholera virus, and Rinderpest) have been weaponized at one time in the past and evaluated under field conditions (15). Since many of the animal commodity groups, including poultry, are clustered in high concentrations in various regions of the country, the possibility of an event of widespread introduction is high. The reasons for potential purposeful introductions are many and varied, and unfortunately it is not difficult to think of scenarios resulting in purposeful introduction of biological agents into the poultry industry. Thus, we must remain vigilant. The regulatory agencies that would respond to introduction of foreign animal diseases are likely capable of handling a single introduction (5), although the recent FMD outbreak in the United Kingdom clearly illustrates the potential devastating effects of just a single entry point of a highly infectious foreign animal disease. A concerted attack on U.S. poultry with multiple introductions would almost certainly paralyze the industry even with the best efforts of the regulatory agencies.

## AVIAN INFLUENZA A VIRUSES AS POTENTIAL BIOWEAPONS

Highly pathogenic avian influenza viruses are generally found on all the lists of potential agricultural bioweapons. Like virulent Newcastle disease viruses that have been weaponized in the past, the AI viruses can be highly and rapidly infectious via respiratory transmission. Unlike Newcastle disease virus, however, they can further be genetically reassorted in the laboratory to combine genes in a grouping that does not exist in nature, either by mating live viruses or rescuing virus from expression plasmids (4,9). The latter plasmid-based techniques are amazing technical developments, but they raise the possibility of major genetic manipulation of viruses and introduction of foreign genes into influenza gene backbones. Consequently, zoonotic influenza viruses in general are getting more attention as potential bioweapons (12). Although it seems highly unlikely that anyone would engineer an avian influenza virus for the purpose of attacking the poultry industry, other potential illegal uses that could spread live or engineered viruses exist. For example, anecdotal evidence exists purporting that poultry farmers have used infectious virus collected from an outbreak to infect their own stock, in attempts to vaccinate or in hopes of indemnification as a result of having infected flocks.

Whatever the case, the need for the capability to rapidly and accurately detect avian influenza viruses, as well as other highly infectious poultry pathogens in the environment, is growing, and new research efforts are needed to evaluate the best approaches to put into the hands of early responders to a purposeful introduction. Availability of validated, rapid, and reliable tests that would supplement the use of slower culture-based detection and immunological subtyping of avian influenza strains would be most useful.

## RAPID DETECTION AND DIAGNOSTICS

In the face of a scenario where multiple purposeful introductions of avian influenza virus into the poultry industry have occurred, rapid and accurate evaluation of environmental contamination becomes critical, especially if a zoonotic virus is encountered. In such a case the following prioritized attributes of a detection assay would be 1) speed and accuracy, 2) simplicity, 3) common platform for both environmental detection and diagnosis of infected animals, and 4) cost. There are nearly as many approaches to rapidly detecting pathogens and diagnosing disease as there are companies and laboratories developing the technologies. The term diagnostics is widely used to refer to both pinpointing a disease based on the presence of the organism in the host and simply detecting the agent where it should not be. In the veterinary world, generally speaking, the early responders will be state or company veterinarians who will see the disease first, then regulatory agencies that will seek to evaluate the extent of presence or spread. In the case of a zoonotic agent, of course, the public health agencies would become involved quickly. In the case of an agricultural pathogen that does not affect human health, contingency plans are in place to control the spread of disease that depend on the nature of the outbreak. Everyone agrees that the faster the pathogen is detected and the extent of contamination of the environment ascertained, the faster the outbreak can be controlled.

The long-sought, magic, 5-min test for detection is most closely approximated by the antigen capture/enzyme-linked immunosorbent assay (ELISA) based approach, where a sample is loaded onto a filter to which specific antibody is bound and a secondary reporter reagent gives colorimetric verification of presence of an antibody-antigen complex. The Directigen® Flu kit (Becton Dickinson, Franklin Lakes, NJ) is currently being used as a screening tool in disease outbreaks for the detection of any influenza A virus. This technology is rapid and has been used for both humans and poultry for years. It is based on detection of the influenza A nucleoprotein and is not suitable for subtyping strains. The sensitivity of such immunologically based tests is generally lower than nucleic acid based tests, and costs are such that individual bird samples must be pooled before screening.

A number of novel rapid detection approaches employ mass spectrometry (MS) to measure ionization and ion capture profiles following treatment of samples (2). The idea is that environmental samples containing pathogens when treated will yield signature patterns that will instantaneously identify the presence of the pathogen. One of these methods, matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) spectroscopy employs a laser to ionize the sample and then a measuring chamber to measure the characteristics of the ions as they speed through the chamber. These instruments are continuously being made smaller and smaller, and suitcase size versions

are currently being evaluated. Speed of detection is the advantage here if the problems associated with dirty environmental samples can be overcome by preselecting the pathogen out of its native matrix. The procedure will also lend itself to chip-based microarray technology since the laser could move over the array and the time of flight measurements made virtually instantaneously. The future success of such a device in the hands of the early responder will depend on working out the problems associated with preparing the environmental sample for clean measurements by the mass spectrometer. Further, while it is likely that an influenza A virus would provide a characteristic or signature MS profile, the possibilities for obtaining strain-specific characteristics are unknown but would seem very unlikely.

Nucleic-acid based detection and diagnostics will ultimately provide the most information to early responders, scientists, and the regulatory agencies. Just as in DNA forensic analysis in humans, characteristic genetic profiles or gene sequences of microbes can be obtained for each individual species and any strain of that species. Rapid detection of pathogens using PCR amplification of specific genes has been employed for many years, but it requires preparation of an electrophoretic gel run with molecular weight standards to identify and quantify the production of the amplicons. For eukaryotes and DNA containing pathogens, restriction fragment length polymorphisms (RFLPs) and many variations on that idea have been used to unequivocally identify organisms. The same approach can be taken with RNA viruses such as influenza viruses (11), and there are commercially available mobile labs that exploit this approach, such as mobile molecular laboratory (model MML-0150, MJ Research, Waltham MA). Ultimately, small scale, suitcase size nucleic acid sequencers will be available, and this will provide the most information of all to identify environmental pathogens. But this technology is not yet available and is certainly not affordable.

There is one nucleic acid based detection approach for which portable platforms are commercially available and which is becoming accepted by early responders (3) as a way to identify environmental pathogens, fluorescent real-time/PCR analysis (FRT/PCR). The fundamental chemistry and reaction conditions for this approach have actually been around for years (7,10) and was originally termed Taqman® PCR (6). Many technological variations of the Taqman chemistry have been developed, including Fret probes and molecular beacon probes, but all use fluorescent probes coupled to PCR for the detection of a wide variety of pathogens. The TAQMAN reaction is extremely specific, more so than other PCR, in that it requires correct alignment of three separate stretches of sequences in the target pathogen. Like PCR, two primers are designed based on specific target sequences and these amplify a short region of the genome in the regular hot/cool cycle of the PCR. In addition, a probe is designed to bind specifically to the amplicons, and this probe contains a fluorescent molecule on one end and a quencher molecule on the other end. The native exonuclease activity of the polymerase in the PCR cleaves this fluorescent molecule during the reaction releasing its fluorescence from the quenching molecule. So as more amplicon is produced, more probe is bound and more fluorescence is released in real time.

What has happened in the last couple of years is the emergence of commercially available portable machines that can use this technology for a host of pathogens. In addition, the availability of dried or packaged reagents for a variety of pathogens will allow early responders to prepare a sample in the field and run FRT/PCR in place. We have prepared a number of primers and probe reagents that are specific for avian influenza viruses, and these are reported and described by Spackman *et al.* in this publication.

The advantages if this new technology and reagents are multiple. One is that the reaction is dead end; that is, after the amplification and measurement, the unopened reaction tube is thrown away, reducing the potential for cross contamination. Second, there are now a variety of fluorescent tags of different wavelengths so that the possibility exists for multiplex analysis within the same reaction tube using different wavelength filters to discern positive reactions. Finally, subject matter experts can analyze the reactions in real time in a format that allows for immediate evaluation over the Internet, since web-enabled software is available for some of the portable systems.

Two commercially available portable systems currently exploit these reactions. They are the Idaho technology ruggedized advanced pathogen identification device (RAPID®) system and the Cepheid Corporation SmartCycler®. Benchtop laboratory versions of each are also available from science supply companies. Each portable machine has desirable features that make it different from the other, but rather than go through those here, I refer the reader to the websites for each company: www.idahotech.com and www.cepheid.com. One

Fig. 1. Handheld PCR devices. Two next generation fluorescent real-time PCR devices. On the left is the handheld advanced nucleic acid analyzer (HANAA), a prototype device developed by Lawrence Livermore Laboratories. The device is battery operable and has four cycling chambers to hold PCR reaction tubes. The unit on the right is Idaho Technology's RAZOR system, which cycles temperatures for the reaction by moving reaction mixtures back and forth in the plastic tubes between fixed temperature chambers and can run 12 reactions simultaneously. In each case, the nucleic acid has to be first extracted from the sample matrix before running the FRT/PCR. With the RAZOR system a set of prepackaged extraction reagents is provided in syringes, and the final sample is injected into the blue plastic receiver tubes. Photos courtesy of Jim Higgins (left) and Idaho Technology (right).

feature of the RAPID system that makes it particularly attractive is its web-enabled software and potential for wireless transmission that allows transmission of data in real time from the field back to a command center. An epidemiologic tracking system developed in concert with the U.S. Air Force, lightweight epidemiology advanced detection and emergency response (LEADER) uses the RAPID system and allows a central command point to be connected to several machines at once deployed at remote sites at considerable distances apart. So, in terms of potential for early responders, these kinds of systems provide a lot of promise. With standardized reagents and extraction protocols and communications with a central command post, evaluation of environmental contamination and infection of animals can be quickly evaluated for the regulatory decision makers.

Fig. 1 illustrates two handheld instruments that use the real-time rapid PCR format and are still in evaluation stages. Efforts continue to miniaturize the detection process such that the technology can be made available to early responders, who would presumably be screening for a single agent to evaluate contamination and spread on the spot. Whether use of these will become a reality in the near future is anyone's guess. Several hundred of the portable RAPID and SmartCycler machines have been sold to various agencies, such as the National Guard and police departments, but the question is whether dried or prepackaged reagents with long enough shelf lives can be made available. FRT/PCR reagents for other RNA viruses similar to influenza A virus have worked in the prepackaged format, so getting from RNA to real-time analysis is certainly possible. Data presented in this symposium have shown that sensitivity of the FRT/PCR approach for avian influenza viruses could be improved, but

specificity is very good and costs promise to be cheaper than the immunologically based detection kits. More research is certainly needed to provide prepackaged reagents that could be used by early responders to detect influenza viruses in the environment. However, given the commercial success of this technology, which was used quite extensively during the anthrax attack in the fall of 2001, it is definitely worth pursuing avian influenza specific primer and probe development and validation of assays in real-world settings, such as those described in this symposium.

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